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IL-10 plays a pivotal role in anti-inflammatory effects of resveratrol in activated microglia cells

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Abstract

The development of agents that can modulate microglial activation has been suggested as one potential strategy for the treatment or prevention of neurodegenerative diseases. Among these agents, resveratrol, with its anti-inflammatory action, has been described to have neuroprotective effects. In this paper we demonstrate that in LPS-stimulated microglia resveratrol pretreatment reduced, in a dose-dependent manner, pro-inflammatory cytokines IL-1β, TNF-α and IL-6 mRNA expression and increased the release of anti-inflammatory interleukin (IL)-10. Moreover, resveratrol pretreatment up-regulated the phosphorylated forms of JAK1 and STAT3, as well as suppressor of cytokine signaling (SOCS)3 protein expression in LPS activated cells, demonstrating that the JAK–STAT signaling pathway is involved in the anti-inflammatory effect exerted by resveratrol. By supplementing the cultures with an IL-10 neutralizing antibody (IL-10NA) we obtained the opposite effect. Taken together, these data allow us to conclude that the LPS-induced pro-inflammatory response in microglial cells can be markedly reduced by resveratrol, through IL-10 dependent up-regulation of SOCS3, requiring the JAK–STAT signaling pathway.

Keywords
MicrogliaResveratrolIL-10InflammationNeurological diseases

1. Introduction

Microglia are the resident phagocytic cells of the central nervous system (CNS), accounting for 20% of the total glial population and as such, being the first line of immune defense in the brain. When stimulated, microglial cells react and produce inflammatory mediators able to coordinate an immune adaptive response. Depending on the predominance of factors secreted, microglia have classically been characterized, similarly to macrophages, as M1 (pro-inflammatory) or M2 (anti-inflammatory) cells [1]. Polarization of macrophage populations toward different phenotypes at different stages of injury might account for this dual role [2], [3], [4]. Recently, the last highlights provide a new descriptive scheme based on the stimulation type and adopting a nomenclature linked to the activation standards (i.e. M(IL-4), M(Ig), M(IL-10), M(GC), M(IFN-γ), M(LPS)) together with the presence or absence of expression marker combinations to ascribe activation

outcomes [5]. In contrast to peripheral macrophages, the mechanisms by which the microglial phenotype is regulated in the CNS are yet poorly understood. Microglia can develop a range of functional phenotypes that broadly correspond to M1/M2 activation of macrophages. Therefore M1 polarized microglia can produce pro-inflammatory cytokines, reactive oxygen species, and nitric oxide, suggesting that these molecules contribute to the dysfunction of neural network in the CNS and, alternatively, M2 polarized microglia express cytokines and receptors that are implicated in inhibiting inflammation and restoring homeostasis [6]. In this respect, M1 activation causes the release of high levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β [7]. The excessive production of inflammatory mediators in the brain can have severe effects and promote neurotoxicity [8].

Evidence supporting the involvement of inflammatory mediators in neurodegeneration in which microglia play a key role is well documented. In this regard, a number of studies have reported that microglial activation is closely associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [9], [10], [11], [12], [13], [14].

Thus, the development of agents that can modulate microglial activation has been suggested as one of the potential strategies for the treatment or prevention of neurodegenerative diseases [15].

There are various microglial activating agents that may ultimately induce neurotoxicity. Among these, lipopolysaccharide (LPS), a bacterial cell wall product of gram-negative bacteria, is widely used to simulate infection under experimental conditions [16], [17].

Resveratrol (3,4,5-trihydroxy-trans-stilbene; Fig. 1), a natural polyphenol present in a variety of medicinal plants, in grapes and in red wine, is widely reported to have neuroprotective properties both in vitro and in vivo. It is also suggested to have anti-inflammatory effects in several systems, including in activated microglia [18], [19], [20]. In addition, resveratrol can cross the blood–brain barrier [21], [22] and modulate some of the symptoms of debilitating neurological disorders, such as ischemia, PD, AD and Huntington's disease [23], [24], [25], [26].
IL-10 is a classic immunoregulatory and anti-inflammatory cytokine. Several authors have shown the inhibitory effect of IL-10 treatment on the production of pro-inflammatory cytokines by reactive glia in response to LPS [27], [28], [29]. It is noticeable that M2 phenotype of activated microglia is able to release high levels of anti-inflammatory cytokines, such as IL-10, correlated with neuroprotection, recovery, and repair in various neurodegenerative diseases [30], [31], [32].

Against this background, the present study was designed to investigate whether the anti-inflammatory effects of resveratrol in the LPS-stimulated N13 microglial cell line may be attributable to up-regulation of the anti-inflammatory cytokine IL-10.

2. Materials and methods

2.1. Cell cultures and treatments

Despite behavioral differences between microglia in vivo and in vitro, cultured microglia offer a powerful tool for testing the potential anti-inflammatory effect of certain compounds and how they can modulate microglial activation [33]. Therefore, for this study we used the N13 microglial cell line, kindly provided by Dr. Ferrari D. (University of Ferrara, Italy). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, referred to as complete medium (Life Technologies-Invitrogen, Milan, Italy). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2 and expanded in tissue culture flasks, changing the medium daily. Then, N13 microglial cells were plated, at a density of 25 × 104/well in 6-wells plates (Falcon) and pre-treated with different concentrations of resveratrol for 1 h before being

treated with LPS (100 ng/mL) for 24 h. For the experiments we used 1–20 μM resveratrol, a concentration range previously shown to be effective in vitro [34], [35], [36], [37]. In a group of experiments IL-10 neutralizing antibody (IL-10NA) (5 μg/mL) was added to microglia cultures to assay the possible involvement of IL-10 in the modulation of microglia inflammatory responses by resveratrol.

Untreated cells were used as controls. Cell viability was determined by the MTT reduction assay, as previously described [38].

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real-time PCR analyses
Total RNA was isolated from cells using Trizol Reagent according to the manufacturer's protocol. The mRNA levels of various genes were quantified using the SYBR Green QuantiTect RTPCR Kit (Roche, South San Francisco, CA, USA). GAPDH was used as endogenous reference. Data were analyzed using the relative standard curve method according to the manufacturer's protocol. The mean value of each gene after GAPDH normalization at the time point showing the highest expression was used as a calibrator to determine the relative levels of TNF-α, IL-1β, IL-6, and IL-10, at different time points. The primer sequences for the tested genes are reported in Table 1.

2.3. Immunofluorescence
Cells were grown on glass coverslips and fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.3% Triton X-100, blocked with goat serum and incubated with anti-IL-10 antibody (1:200 AbCam, DBA, Milan, Italy) overnight at 4 °C. After washing, the cells were incubated with goat anti-mouse IgG labeled with Alexa Fluor 488 (1:200) (Molecular Probes, Invitrogen, Milan, Italy), for 2 h at 37 °C. The nuclei of cells were counterstained with 49,6-diamidino-2-phenylindole (DAPI) (0.1 mg/mL, Sigma). Alexa Fluor 488 was excited at 488 nm and then detected between 506–538 nm. Fluorescence intensity of cells was observed with a Nikon Eclipse i80 and the images were captured with a photocamera.

2.4. ELISA
IL-10 levels in cell culture supernatants were also quantitatively determined by an enzyme-linked immunosorbent assay (ELISA) (Invitrogen Technology, Milan, Italy), as described in the manufacturer's instructions, and expressed as pg/mL. IL-10 quantification was performed in the supernatants of the cultures at 24 h after cell treatments. All determinations were made in triplicate.

2.5. Electrophoresis
After treatment of cultures as previously described, cells were harvested and lysed by ice-cold lysis buffer [1% Triton X-100, 20 mM Tris–HCl, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin hemisulfate salt, 0.2 U/mL aprotinin (all from Sigma-Aldrich)] for 30 min on an ice-bath. The lysate was vortexed for 15–20 s and then centrifuged at 12 800 ×g for 20 min; the protein concentration in the supernatant was spectrophotometrically determined by Bradford's protein assay [39], protein samples were diluted with sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 10% w/v SDS, 5% β2-mercaptoethanol, 0.05% w/v bromophenol blue) and then boiled for 3 min. Proteins (25 μg/lane) and prestained standards (BioRad Laboratories, Hercules, CA, USA) were loaded on 7% or 12% SDS precast polyacrylamide gels (BioRad Laboratories).

2.6. Western blotting
After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blotting buffer [20 mM Tris/150 mM glycine, pH 8.0, 20% (v/v) methanol] was used for gel and membrane saturation and blotting. Then, membranes were incubated in the dark with 1 μg/mL mouse monoclonal antibody (mAb) anti-JAK1 (1:100), anti-STAT3 mAb (1:100), anti-phospho-JAK1 mAb (1:100) or anti-phospho-STAT3 mAb (1:100) and mouse polyclonal Ab anti β-actin (1:500) (all from Santa Cruz Biotechnology, DBA Italia, Milan, Italy), for 60 min at room temperature. The membranes were washed

with 0.1% Tween 20-PBS (for 20 min, 3 times) and then incubated with 1:10,000 diluted horseradish peroxidase (HRP)-conjugated anti-mouse (Santa Cruz Biotechnology) for 60 min. Bands were visualized by chemiluminescence detection (Invitrogen, Milan, Italy). The β-actin level was used as protein loading control. All data were obtained in triplicate independent experiments.

2.7. Densitometric analysis

The bands obtained after immunoblotting were submitted to densitometric analysis using ID Image Analysis Software (Kodak Digital Science). Results were expressed as arbitrary units.

2.8. Data presentation and statistical analysis

Student's t test and analysis of variance (one-way ANOVA) on the results of at least five independent biological replicates were performed. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Viability test

Using the MTT assay, we found that the viability of cells exposed for 24 h to 100 ng/mL LPS was significantly impaired in comparison to controls. Interestingly, resveratrol (1–20 μM) had no detectable toxic effects on N13 cells cultured in the absence of LPS. In addition, the viability of LPS stimulated cells was significantly improved after pre-treatment with resveratrol, in a dose-dependent manner, reaching a maximum level at 10 μM (Fig. 1).

3.2. Resveratrol modulates LPS-induced mRNA expression of cytokines in microglia

RT-PCR was performed to determine whether resveratrol regulates pro-inflammatory cytokine expression at transcriptional level. As shown in Fig. 2, we demonstrated a marked increase in TNF-α, IL-1β and IL-6 mRNA in N13 microglial cells after 24 h of LPS treatment. No effect by treatment with resveratrol alone on pro-inflammatory gene expression was observed in N13 cells, since pro-inflammatory cytokine mRNA levels resulted similar in all resveratrol concentrations (1–20 μM) used and comparable to the levels detected in control cells. Interestingly, we observed that mRNA expression of pro-inflammatory cytokines was significantly decreased in LPS-treated cells after pretreatment with resveratrol (1 to 20 μM) in comparison to microglial cells stimulated with LPS alone, suggesting that, in LPS-activated cells, resveratrol was able to exert a negative effect on pro-inflammatory cytokine mRNA expression, in a dose-dependent manner, reaching a maximal reduction at 10 μM (Fig. 2).

RT-PCR was also performed to determine whether resveratrol was able to regulate the anti-inflammatory cytokine IL-10 at the transcriptional level. As shown in Fig. 3A, LPS induced an increased IL-10 mRNA in microglial cells. We also observed detectable levels of IL-10 mRNA in microglial cells pre-treated with resveratrol alone; maximal levels were obtained in the presence of 10 μM resveratrol. Interestingly, levels of IL-10 mRNA resulted significantly higher in LPS-treated N13 cells previously submitted to resveratrol treatment, demonstrating that resveratrol was able to modulate IL-10 expression at transcriptional level. Maximal upregulation of mRNA IL-10 expression in LPS-activated cells was observed at 10 μM resveratrol, as reported in Fig. 3A.

3.3. Effect of resveratrol on IL-10 release

IL-10 release in culture supernatants was also assessed by the ELISA test both in the absence and presence of resveratrol. As shown in Fig. 3B, 100 ng/mL of LPS treatment significantly increased IL-10 concentrations in culture supernatants. Interestingly, at the doses tested in this study resveratrol significantly increased IL-10 production, implying that resveratrol could also exert its anti-inflammatory effect by positively modulating the expression of IL-10. Although all concentrations tested were able to increase IL-10 production, in comparison to controls, we observed that 10 μM resveratrol resulted in the most effective concentration inducing the maximal release of IL-10. Morphological observations demonstrating IL-10 production were confirmed by immunofluorescence test, as reported in Fig. 3C. N13 cultures exhibited a

weak but significant fluorescence compared to controls when stimulated with LPS or resveratrol alone. However, cells submitted to combined treatment with resveratrol and LPS showed a significant overexpression of IL-10, as demonstrated by significantly higher levels of fluorescence (Fig. 3C).

3.4. mRNA expression of inflammatory mediators in the presence of exogenous IL-10NA

To investigate the involvement of IL-10 in the modulation by resveratrol of microglia inflammatory responses, we added exogenous IL-10NA to the cultures treated with resveratrol.

This approach allowed us to elucidate whether IL-10 plays a role in the anti-inflammatory property of resveratrol in microglia cells. As shown in Fig. 4, IL-10NA strengthened the expression of the TNF-α, IL-1β and IL-6 transcripts when added to LPS-stimulated cells in the presence of resveratrol.

3.5. Effect of resveratrol pretreatment on the expression of STAT3/p-STAT3, JAK1/p-JAK1 and JAK2/p-JAK2 in microglial cells

Then, in order to investigate the mechanism underlying these resveratrol anti-inflammatory effects, we further examined the protein levels of STAT3 and p-STAT3 in the N13 cell line. The protein expression of p-STAT3 was significantly increased in the LPS-stimulated cells. Pretreatment with resveratrol efficiently increased, in a dose-dependent manner, the active form of STAT3 (Fig. 5).

We then focused on protein kinases upstream of STAT3 phosphorylation, evaluating the protein levels of JAK1, p-JAK1, JAK2, and p-JAK2, which are the upstream regulation molecules of STAT3. The total proteins of JAK1 and JAK2 were not significantly different in the groups tested. However, the levels of p-JAK1 and p-JAK2 resulted significantly increased in the LPS-treated cells, whereas pretreatment with resveratrol significantly increased, in a dose-dependent manner, the active form of JAK1 but not of JAK2, as reported in Fig. 6.

3.6. Effect of resveratrol pretreatment on the expression of SOCS3 in microglial cells

In addition, we investigated the expression of the negative regulator of cytokine expression, SOCS3, in microglial cells. As expected, we have not observed a significant increase of SOCS3 in LPS-activated cells. However, in activated cells pre-treated with resveratrol we detected a significant increase of SOCS3 protein levels, which resulted dose-dependent, reaching the maximum levels at 10 μM resveratrol, as reported in Fig. 7. Interestingly, we observed that adding IL-10NA to LPS activated cells after pre-treatment with 10 μM resveratrol determined a significant reduction of p-JAK1, p-STAT3 and SOCS3 protein levels, showing that the ability of resveratrol to up-regulate SOCS3 expression is probably due to IL-10 release, through the JAK1/STAT3 signaling pathway (Fig. 8).

4. Discussion

In the present study, we demonstrated anti-inflammatory effects of resveratrol in LPS-activated microglial cells. Collectively, our data showed that resveratrol suppressed, in a dose-dependent manner, LPS-induced expression of TNF-α, IL-1β and IL-6 mRNA, which are key proinflammatory and neurotoxic molecules in microglia. Furthermore, we demonstrated that resveratrol was able to up-regulate, in a dose-dependent manner, both mRNA and protein IL-10 levels, effects that might help to explain neuroprotective ability of resveratrol. Accumulating evidence indicates that resveratrol confers neuroprotection in various animal models of brain disorder (for review see Ref. [19]) and our results are in accordance with other studies described in literature reporting an immunomodulatory activity of resveratrol [40], [41], [42].

We observed that microglia cells submitted to resveratrol treatment are able to increase IL-10 production. One possibility is that resveratrol may act as a polarizing agent in microglia cells, favoring the shifting versus M2 phenotype, more efficient as IL-10 producer. Another possibility is that IL-10 is induced as the predominant effect in these cells, producing an inhibition of proinflammatory cytokines in an autocrine/paracrine fashion as previously reported [43].

Although we are not able to explain how resveratrol up-regulates microglia IL-10 production in our experimental model, similar effects are described in literature, thus confirming our observations [44], [45], [46].

Notoriously, IL-10 has marked suppressive effects on the production of pro-inflammatory cytokines by monocytes–macrophages and downregulates the expression of activating molecules on these cells [47], [48], [49], [50], [51]. IL-10 is a classic immunoregulatory and anti-inflammatory cytokine. In this regard, several authors have reported the inhibitory effect of IL-10 treatment on the production of pro-inflammatory cytokines by reactive glia in response to LPS [27], [28], [29], [52].

In accordance with these findings, our results demonstrated that IL-10NA administration was able to suppress the anti-inflammatory effects of resveratrol in LPS-activated cells, suggesting that resveratrol treatment, by up-regulating IL-10 expression, could potentially be associated with the reduced inflammatory response observed. These conclusions are in agreement with previous studies showing that IL-10 decreases pro-inflammatory cytokine release through a reduction of surface expression receptors for TNF-α, IL-1 and IL-6 [53], [54], [55], [56].

Another point of discussion raised in this work is the effect of resveratrol treatment on JAK1 and STAT3 activation. Resveratrol treatment determined a significant increase of the activated forms of JAK1 and STAT3 in comparison to LPS treated cells, suggesting that the anti-inflammatory effect exerted by resveratrol is due, at least in part, to JAK1/STAT3 pathway modulation. In this regard, IL-10 expression is well known to be dependent on the JAK/STAT pathway in myeloid cells, and IL-10 predominantly activates STAT3, which is mainly involved in the negative regulation of macrophage activation [57], [58]. In fact, when IL-10 binds IL-10R, it activates JAK1, the only JAK family member required for IL-10 signaling [59], [60]. Several reports have claimed that, apart from STAT3, IL-10R activates other STAT proteins and can initiate alternate signaling pathways. However, more definitive studies have shown that STAT3 is the only STAT protein required to induce the anti-inflammatory effects of IL-10 [61], [62]. The JAK–STAT signaling pathway is one of the most important signal transduction cascades and is essential for the regulation of cytokine receptor signaling [63]. STAT3, when phosphorylated by the receptor-associated JAKs, translocates to the nucleus where it binds with a high affinity to the promoters of various IL-10-responsive genes. One of these genes is SOCS3, a member of a well identified family of genes mediating feedback inhibition of cytokine-induced responses, acting as a negative regulator of cytokine signaling and able to suppress pro-inflammatory cytokine activity by inhibiting JAK/STAT-dependent signaling [64].

Then, the ability of IL-10 to induce de novo synthesis of SOCS3 in monocytes is correlated with its ability to inhibit the expression of many genes in these cells, including endotoxin-inducible cytokines such as TNF-α and IL-1. In addition, the capacity of IL-10 to inhibit pro-inflammatory gene expression in monocytes is associated with its ability to rapidly induce the synthesis of SOCS3 [64]. This is also supported by our results showing the ability of resveratrol to increase SOCS3 expression in LPS-activated cells. Thus, the ability of resveratrol to induce IL-10, which in turn inhibits gene expression in microglia cells through the up-regulation of SOCS3, is probably associated with the anti-inflammatory effects detected in vitro.

Interestingly, we observed that resveratrol treatment was able not only to up-regulate STAT3 activation but also the upstream molecule JAK1. A possible explanation for this effect could be that IL-10 release after resveratrol treatment may activate the JAK1/STAT3 signaling pathway that, in turn, may be responsible for pro-inflammatory gene suppression. This hypothesis is confirmed by the observation that neutralizing IL-10 activity with IL-10NA reverted the anti-inflammatory effect of resveratrol treatment, restoring pro-inflammatory gene expression to the levels observed in cells treated with LPS alone, in the absence of resveratrol. Finally, IL-10NA treatment of LPS-activated cells in the presence of resveratrol determined a

significant reduction of JAK1/STAT3 activation and a reduced SOCS3 expression. On the basis of these observations, a mechanism can be proposed whereby resveratrol inhibits LPS-induced STAT3 activation in microglia cells through IL-10-dependent SOCS3 upregulation (Fig. 9), thus explaining its down-regulatory effects on pro-inflammatory cytokine mRNA expression. Further investigations, using primary microglia or in vivo conditions, including knockout animal models, may be useful to confirm data obtained in this study. In conclusion, we demonstrate, for the first time in LPS-activated microglial cells, that resveratrol is able not only to attenuate pro-inflammatory events, but also to promote an anti-inflammatory response, in terms of IL-10 upregulation which, in turn, increases SOCS3 expression through a JAK1/STAT3-dependent pathway. Therefore, the strong anti-inflammatory effects of resveratrol may offer a potential treatment for neurodegenerative diseases accompanied by microglial activation.

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Fig.1

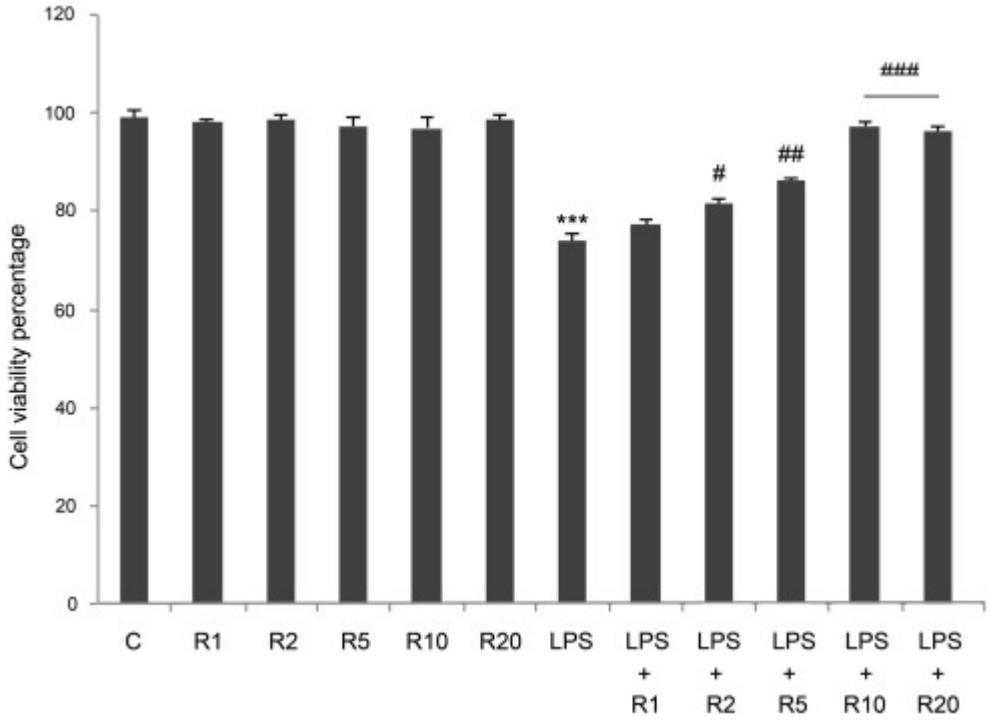

Fig. 1. Effects of resveratrol and LPS treatment on cell viability. N13 microglial cells were treated with various concentrations (1, 2, 5, 10, 20 μM) of resveratrol (R) alone or in the presence of 100 ng/mL LPS for 24 h. Untreated cells were used as control (C). Cell viability was examined using the MTT test. Experimental treatments were analyzed in triplicate and data are expressed as the mean ± SD of five independent experiments. ***$p < 0.001$ LPS vs C; ###$p < 0.001$ LPS + R vs LPS; ##$p < 0.01$ LPS + R vs LPS; #$p < 0.05$ LPS + R vs LPS.

Table 1

Gene	Sequence
GAPDH forward primer	5′-ACCACAGTCCATGCCATCAC-3′
GAPDH reverse primer	5′-TCCACCACCCTGTTGCTGTA-3′
TNF-α forward primer	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′
TNF-α reverse primer	5′-ACATTCGAGGCTCCAGTGAATTCGG-3
IL-1β forward primer	5′-CGCAGCAGCACATCAACAAGAGC-3
IL-1β reverse primer	5′-TGTCCTCATCCTGGAAGGTCCACG-3
IL-6 forward primer	5′-TTCTTGGGACTGATGCTG-3′
IL-6 reverse primer	5′-CTGGCTTTGTCTTTCTTGTT-3′
IL-10 forward primer	5′-GCCAGTACAGCCGGGAAGACAATA-3′
IL-10 reverse primer	5′-GCCTTGTAGACACCTTGGTCTT-3′

Table 1. DNA sequences of primers used in PCR reactions.

Fig.2

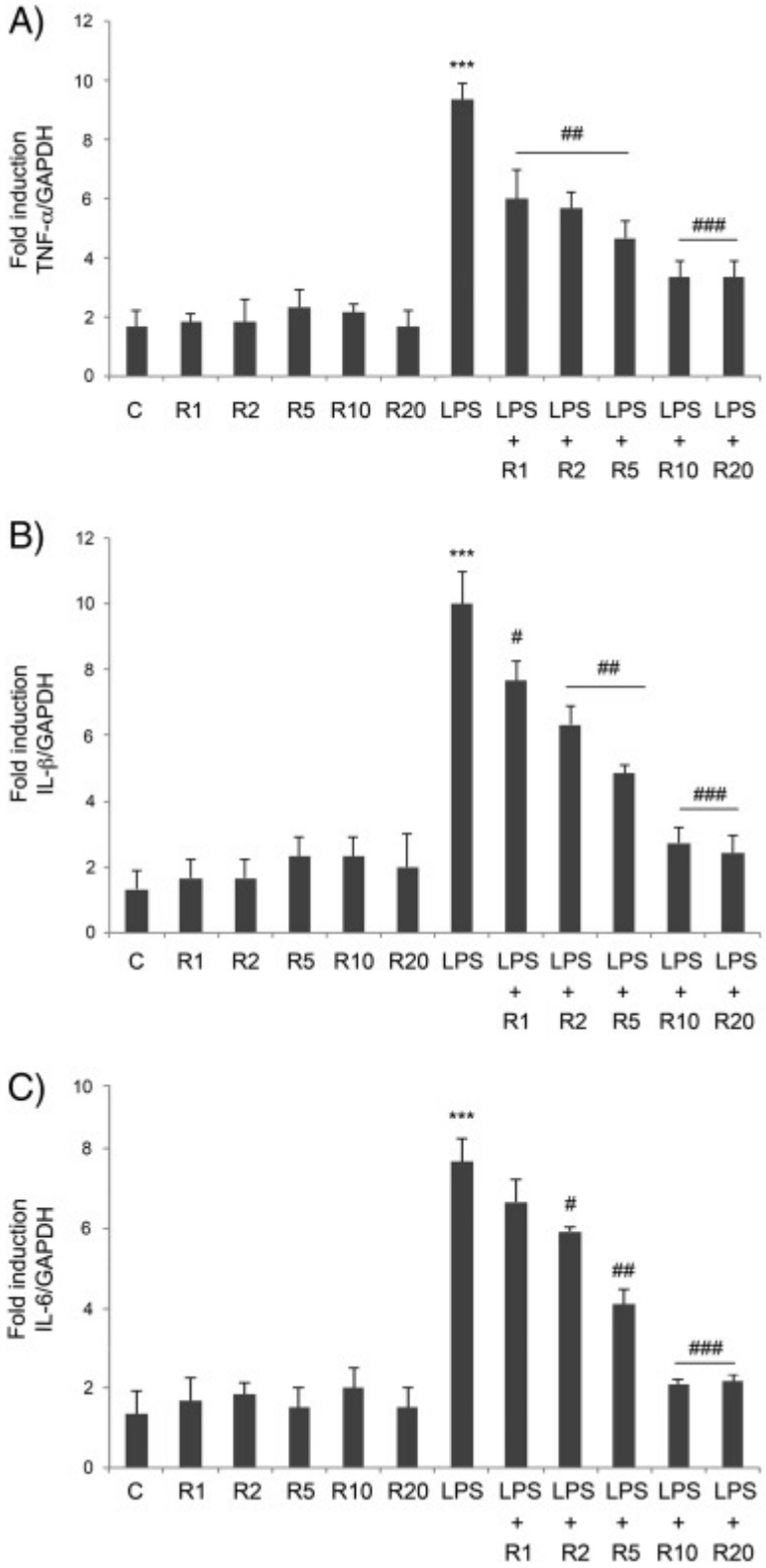

Fig. 2. Effect of resveratrol on pro-inflammatory cytokine mRNA expression determined by real-time RT-PCR. (A) TNF-α mRNA expression; (B) IL-1β mRNA expression; (C) IL-6 mRNA expression. N13 microglial cells were treated with various concentrations (1, 2, 5, 10, 20 μM) of resveratrol (R) alone or in the presence of 100 ng/mL LPS for 24 h. Experimental treatments were analyzed in triplicate and data are expressed as the mean ± SD of five independent experiments. ***$p < 0.001$ LPS vs C; ###$p < 0.001$ LPS + R vs LPS; ##$p < 0.01$ LPS + R vs LPS; #$p < 0.05$ LPS + R vs LPS.

Fig. 3

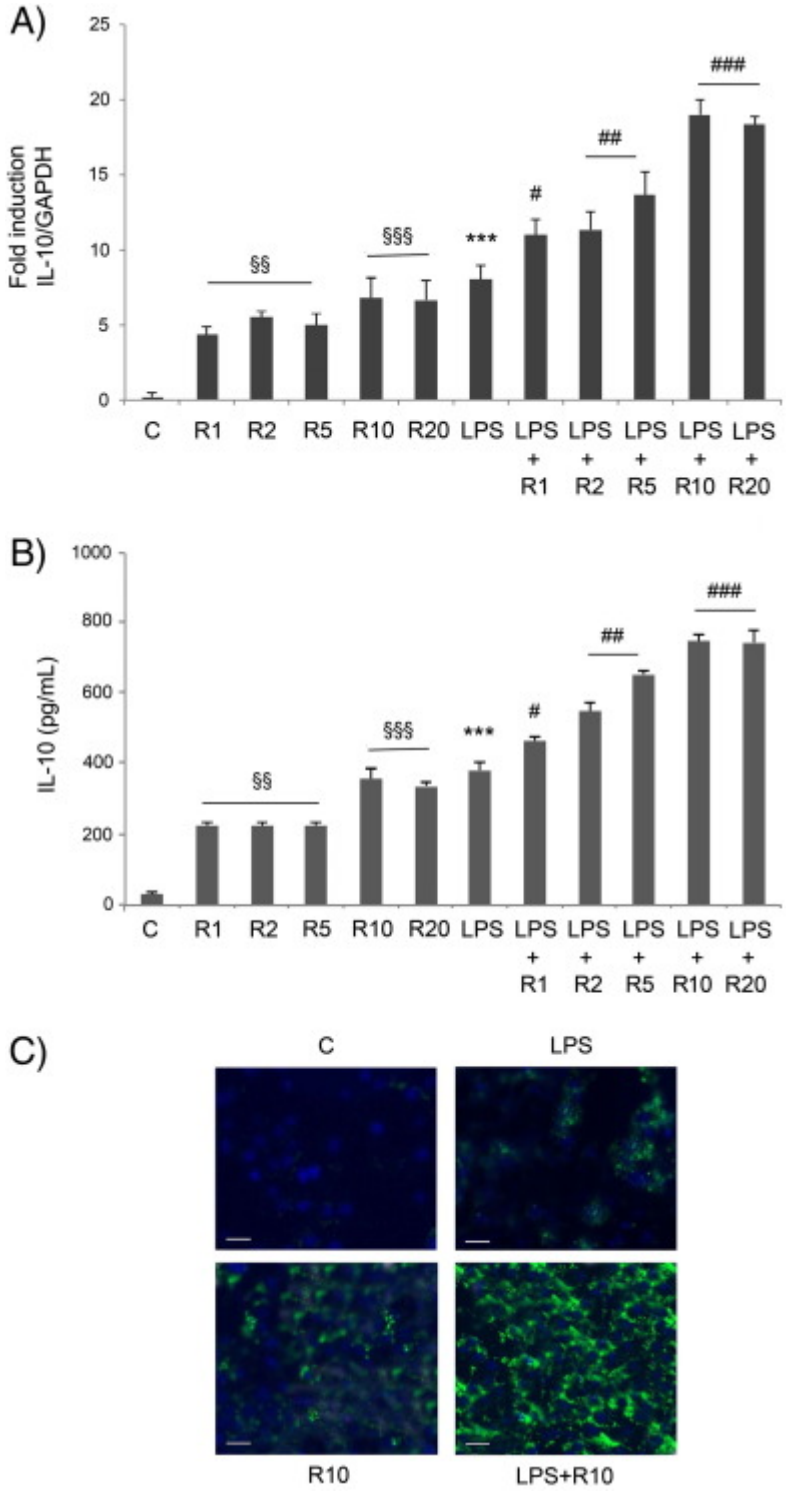

Fig. 3. Effect of resveratrol on intracellular IL-10 release. (A) IL-10 mRNA expression determined by real-time RT-PCR; (B) IL-10 release determined by ELISA test; (C) representative images of the fluorescence for intracellular IL-10 formation (480 nm excitation, 527 nm emission). N13 microglial cells were treated with various concentrations (1, 2, 5, 10, 20 μM) of resveratrol (R) alone or in the presence of 100 ng/mL LPS for 24 h. Scale bar 20 μm. Experimental treatments were analyzed in triplicate and data are expressed as the mean ± SD of five independent experiments. §§$p < 0.01$ R vs C; §§§$p < 0.001$ R vs C; ***$p < 0.001$ LPS vs C; ###$p < 0.001$ LPS + R vs LPS; ##$p < 0.01$ LPS + R vs LPS; #$p < 0.05$ LPS + R vs LPS.

Fig. 4

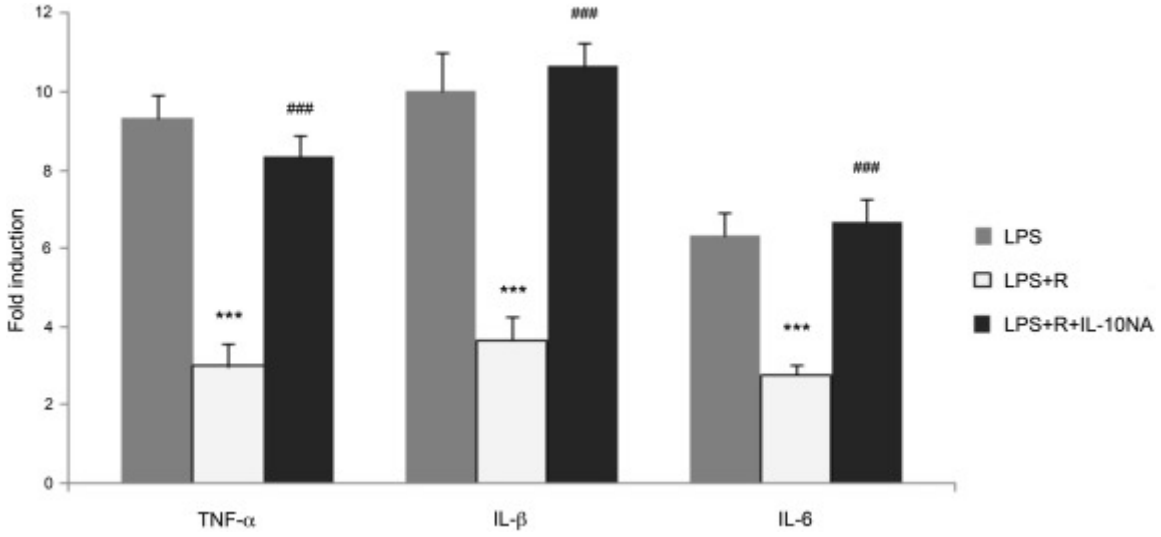

Fig. 4. Effect of resveratrol (R) and IL-10NA on pro-inflammatory cytokine mRNA expression determined by real-time RT-PCR. N13 microglial cells were incubated for 24 h with 100 ng/mL LPS alone, or after pre-treatment with 10 μM of resveratrol alone and in combination with both 10 μM of resveratrol and 5 μg/mL of IL-10NA. This assay was performed in triplicate and data are expressed as the mean ± SD of five independent experiments. ***p < 0.001 LPS + R vs LPS; ###p < 0.001 R + LPS + IL-10NA vs LPS + R.

Fig. 5

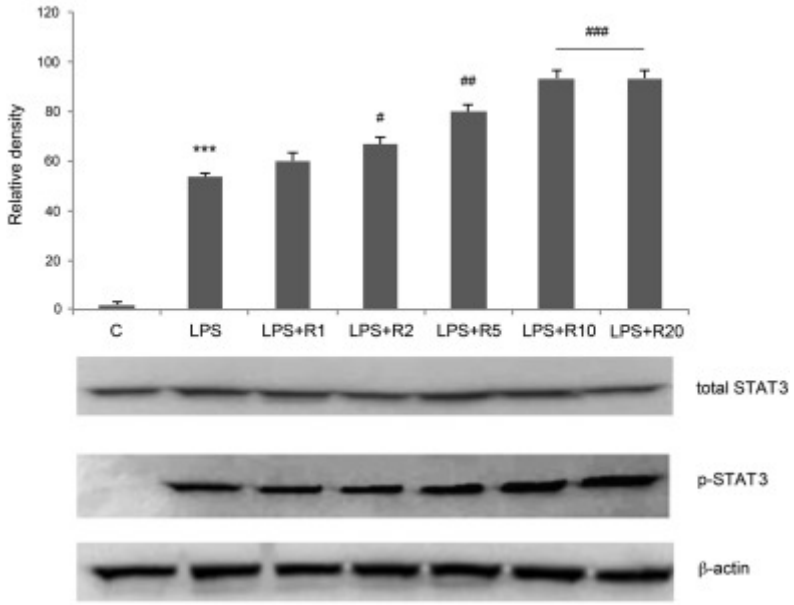

Fig. 5. Effects of resveratrol (R) and LPS on STAT3 expression determined by immunoblot analysis of STAT3 and p-STAT3. N13 microglial cells were treated for 24 h with 100 ng/mL LPS alone or in the presence of various concentrations (1, 2, 5, 10, 20 μM) of resveratrol (R). Untreated cells were used as controls. Protein expression levels were normalized to β-actin and results of densitometric analysis are expressed as means ± SD of five independent experiments. ***p < 0.001 LPS vs C; #p < 0.05 LPS + R vs LPS; ##p < 0.01 LPS + R vs LPS; ###p < 0.001 LPS + R vs LPS.

Fig. 6:

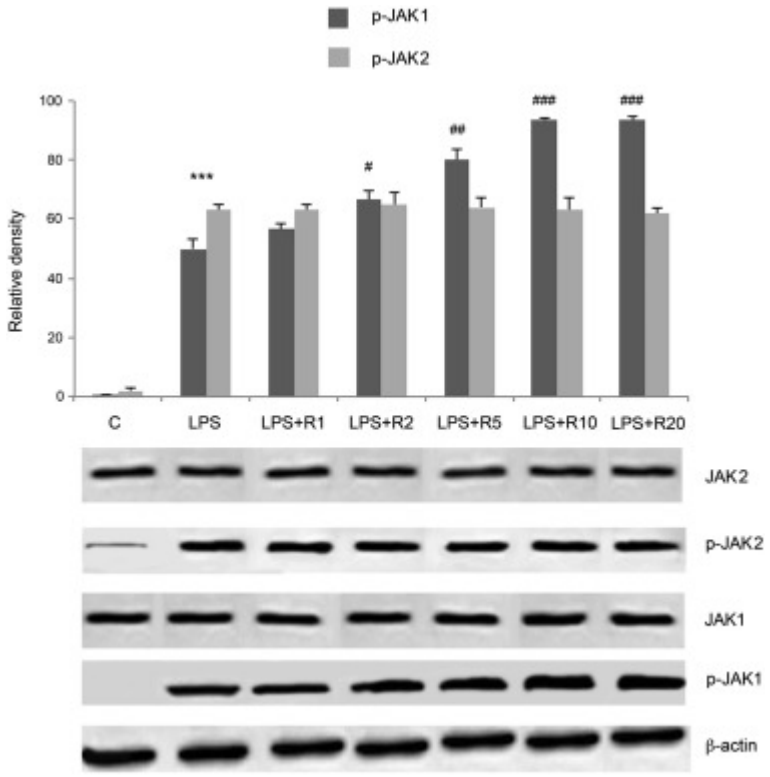

Fig. 6. Immunoblot analysis of JAK1, p-JAK1, JAK2 and p-JAK2 expression. N13 microglial cells were treated for 24 h with 100 ng/mL LPS alone or in the presence of various concentrations (1, 2, 5, 10, 20 μM) of resveratrol (R). Untreated cells were used as controls. Protein expression levels were normalized to β-actin and results of densitometric analysis are expressed as means ± SD of five independent experiments. ***p < 0.001 LPS vs C; #p < 0.05 LPS + R vs LPS; ##p < 0.01 LPS + R vs LPS; ###p < 0.001 LPS + R vs LPS.

Fig. 7:

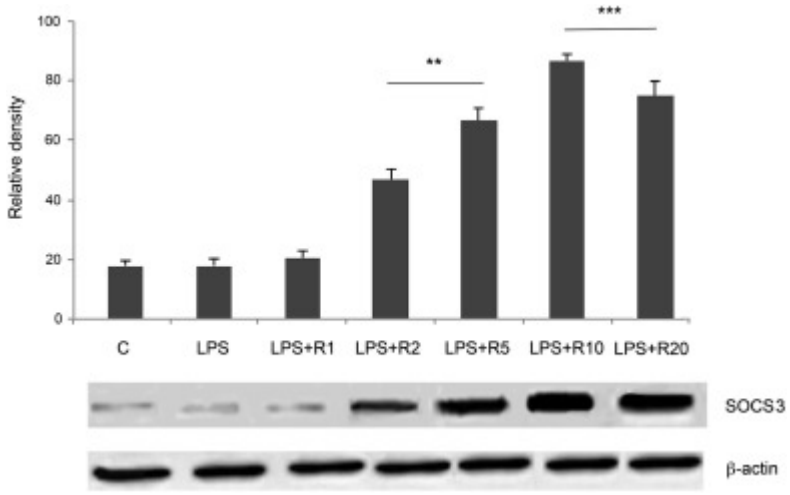

Fig. 7. Effects of resveratrol (R) and LPS on SOCS3 expression determined by immunoblot analysis. N13 microglial cells were treated for 24 h with 100 ng/mL LPS alone or in the presence of various concentrations (1, 2, 5, 10, 20 μM) of resveratrol (R). Untreated cells were used as controls. Protein expression levels were normalized to β-actin and results of densitometric analysis are expressed as means ± SD of five independent experiments. **p < 0.01 LPS + R vs LPS; ***p < 0.001 LPS + R vs LPS.

Fig. 8

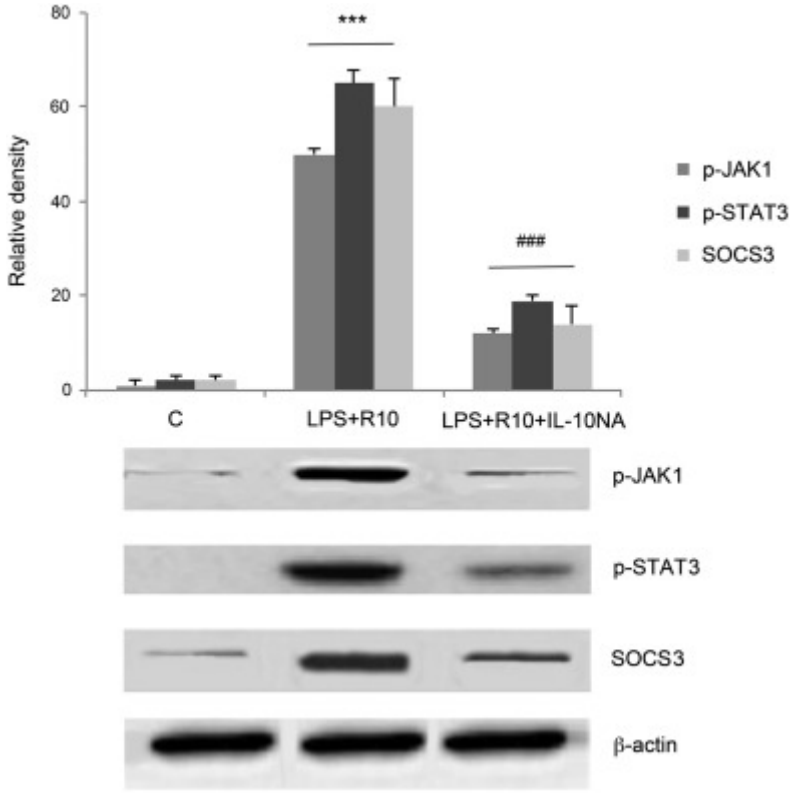

Fig. 8. Effects of resveratrol (R), LPS and IL-10NA on p-JAK1, p-STAT3 and SOCS3 expression determined by immunoblot analysis. N13 microglial cells were incubated for 24 h with 100 ng/mL LPS after pre-treatment with 10 μM resveratrol alone and in combination with 5 μg/mL of IL-10NA. This assay was performed in triplicate and data are expressed as the mean ± SD of five independent experiments. ***$p < 0.001$ LPS + R vs ###$p < 0.001$ LPS + R + IL-10NA vs LPS + R.

Fig. 9:

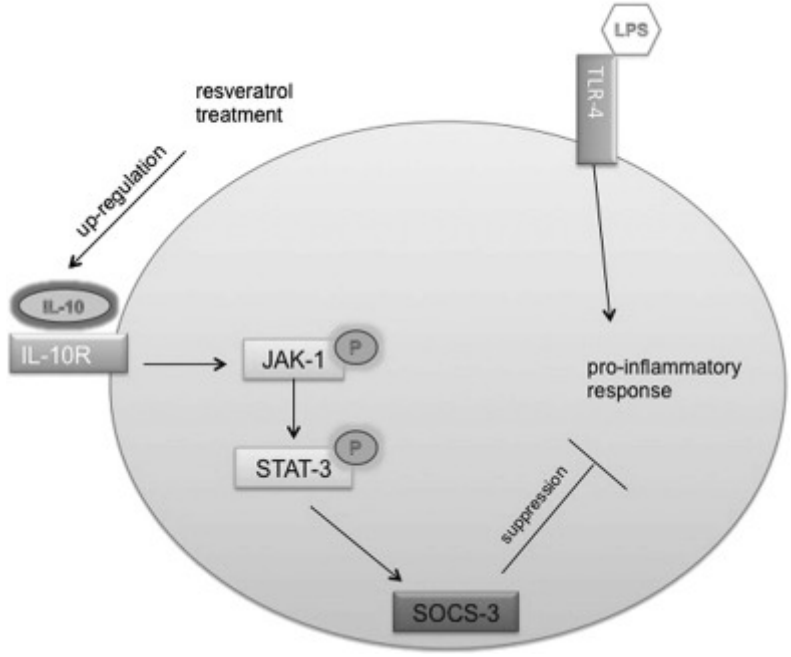

Fig. 9. Proposed mechanism explaining the anti-inflammatory action exerted by resveratrol on LPS-activated microglia cells.